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Synthesis and secretion of Alzheimer amyloid βA4 precursor protein by stimulated human peripheral blood leucocytes

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Alzheimer amyloid precursor proteins (APP) are actively secreted by stimulated human peripheral mononuclear blood leucocytes (PMBLs). Induction of APP transcription, translation and secretion was observed with several T cell mitogens but was highest with phytohemagglutinin. The time course of induction is similar to that reported for IL-2 and IL-2 receptor. We suggest that APP may play an important role in the construction of the immunological network and the differentiation of T cells.

Alzheimer's disease, Amyloid precursor protein; Leucocyte

1. INTRODUCTION

Alzheimer's disease is characterized by the presence of intra- and extraneuronal amyloid protein depositions in the brain of affected individuals. Amyloid appears as neurofibrillary tangles, amyloid plaques and vascular amyloid [1–4]. The amyloid βA4 protein is a small self-aggregating protein of about 42/43 residues that is synthesized as part of a larger, glycosylated transmembrane amyloid precursor protein (APP) [1,3,5]. Transmembrane APP's constitute a family of proteins that are generated from alternatively spliced transcripts (695, 714, 751 or 770 amino acids) of the single APP gene [5–9]. Proteolytic cleavage within the βA4 sequence dissects the extracellular domains of APP from the transmembrane and cytoplasmic domain, and leads to secretion of C-terminally truncated, non-amyloidogenic proteins [10,11]. The secreted forms of APP_{751} and APP_{770}, which contain a 57-amino acid insert with homology to the Kunitz family of protease inhibitors, are protease nexin II [12,13]. Transmembrane as well as secretory APPs are found in brain and peripheral tissues, secretory APPs are also detected in body fluids such as blood and cerebrospinal fluid [14–20].

The identification of secreted APP in human sera from patients with Alzheimer's disease as well as in sera of healthy individuals rises the question for the origin of APP in serum. Several authors could demonstrate that activated platelets can secrete C-terminally truncated APP. Subsequently, it was shown that this APP is derived from $APP_{751/770}$. The latter are natural inhibitors of blood clotting factor XIa suggesting that these APP's play an important role in blood coagulation [21–23].

In this study we investigated another possible origin of APP in the serum. Here we demonstrate that peripheral mononuclear blood leucocytes (PMBL) secrete truncated APP, predominantly the variants containing the trypsin protease inhibitor domain ($APP_{751/770}$), after stimulation by phytohemagglutinin (PHA), pokeweed mitogen (PWM) or concanavalin (ConA). It seems likely that APP is a T cell product since all three mitogens stimulate T cell proliferation and lymphokine production. The extensive expression of APP in stimulated PMBL's indicates that the expression of APP is correlated with certain stages of cell proliferation and cell differentiation.

2. MATERIALS AND METHODS

2.1. *Cell preparation and culture conditions*

Human peripheral mononuclear blood leucocytes (PMBL) were obtained from buffy coats of normal donors (Blood Center, Heidelberg) by Ficoll-Hypaque gradient density centrifugation. The purification was performed essentially after Böyum et al. (1968) [25].

Isolated PMBLs or lymphocytes (monocyte-depleted PMBLs), were cultured at a density of 2×10^6 cells/ml in Dulbecoo's modified Eagle medium (DMEM, Gibco/BRL, UK) containing 1 g/l glucose, penicillin (50 U/ml), streptomycin (40 μg/ml), and 10% (v/v) fetal calf serum (FCS, Gibco/BRL, UK).

Activation of resting lymphocytes was induced by culturing cells in medium supplemented with different mitogens: phytohemagglutinin

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(10 μg/ml), concanavalin A (10 μg/ml) or pokeweed mitogen (5 μg/ml) for different time intervals. Lectins were purchased from Sigma chemicals (St. Louis, MO, USA). Medium was not exchanged during stimulation.

2.2. *Biosynthetic labelling*

After removal of the culture medium by centrifugation, 1×10^7 cells were radioactively labelled with 120 μCi of [^{35}S]methionine in 1.5 ml of methionine-free DMEM. The conditioned medium was then cleared by centrifugation and stored at −20°C. The cells were washed once with phosphate-buffered saline (PBS). For lysis, cells (5×10^6 cells) were resuspended in 0.2 ml lysis buffer [50 mM Tris (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1% Nonidet P40 supplemented with 2 mM phenylmethylsulfonylfluoride (PMSF)] and incubated for 30 min on ice. Cell lysates were centrifuged at 10 000 × *g* for 5 min and the supernatants were stored at −20°C until further use. The extraction pellet was discarded.

2.3. *Immunoprecipitation*

For immunoprecipitation, 100 μl of cell-lysate or 750 μl of conditioned medium were used. The cell-lysate was diluted 1:4 with ice-cooled PBS, the conditioned medium was supplemented with 15 μl medium buffer [1 M Tris (pH 8.0), 100 mM EDTA, 10% Nonidet P40, 100 mM PMSF]. Lysate and medium were preincubated for 2 h at 4°C with 10 μl preimmune serum and 3 mg protein A-Sepharose (Pharmacia, Uppsala, Sweden). The insoluble complexes were spun down and discarded. The supernatants were incubated for 1 h at 4°C with 5 μl of undiluted anti-FdAPP antiserum. Anti-FdAPP is a polyclonal rabbit antiserum raised to purified *E. coli* FdAPP fusion-protein [14]. After addition of 2 mg of protein A-Sepharose the mixture was incubated for 30 min at 4°C. The insoluble complexes were washed three times with washing-buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 0.2% Nonidet P40, 2 mM PMSF) and resuspended in 3 × Laemmli sample buffer. After boiling, labelled proteins were subjected to SDS-PAGE. Gels were soaked in En^{3}Hance (New England Nuclear, USA), dried and exposed to Kodak X-Omat AR film for one week at −70°C.

2.4. *Molecular cloning and S1 nuclease protection assay*

Molecular cloning and S1 nuclease protection assay were performed as described by König et al. [40].

2.5. *Immunohistochemistry*

Cryosections (8 μm) were obtained from a muscle biopsy of a 58-year-old female patient with polymyositis. Endogenous peroxidase was blocked with 1% H_2O_2 methanol for 30 min. Sections were then incubated overnight at room temperature with affinity purified monoclonal anti-APP antibody 22C11 at a concentration of 10 μg/ml [14]. Bound antibody was detected using the avidin-biotin-peroxidase method (Vector, USA) and 3,3-diaminobenzidine as chromogenic substrate. The sections were counterstained with hematoxylin. An irrelevant antibody was used as control.

3. RESULTS

Activated platelets are one possible origin of serum APP [21–23]. Here we show that activated mononuclear blood leucocytes are another source of serum APP. To proof this, PMBLs were isolated from buffy coats of healthy donors and their APP expression was analysed.

To assess the biosynthesis of APP by unstimulated PMBLs, freshly isolated cells were cultured without mitogens in the presence of [^{35}S]methionine. The APP content of cell lysate and conditioned medium was analysed by immunoprecipitation. As shown in Fig. 1a, neither the culture medium nor the cell lysates contain

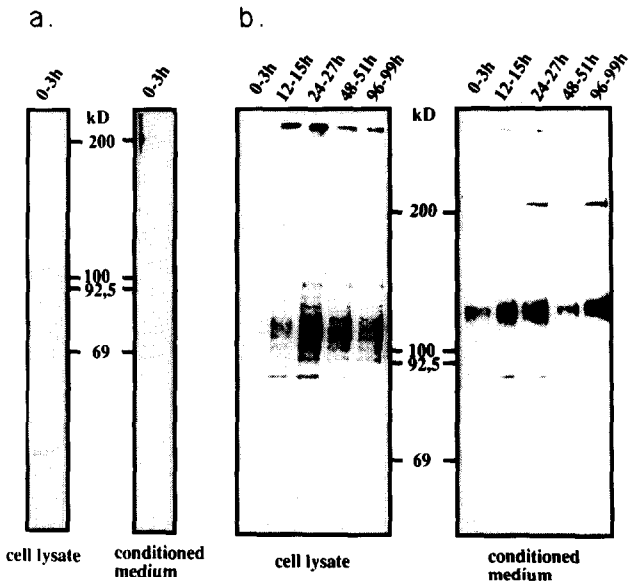


Fig. 1. Time course of APP biosynthesis in PMBLs after PHA induction. Freshly isolated PMBLs were incubated in the absence (a) or in the presence of PHA (10 μg/ml) (b). Biosynthetic labelling with [^{35}S]methionine was done at times indicated at the top of each lane. Cells and conditioned medium were subjected to immunoprecipitation with anti-FdAPP. The autoradiography of the immunoprecipitates is shown (8% SDS-PAGE).

APP. These data suggest that unstimulated PMBLs do not produce amyloid precursor proteins, or that they produce APP at such low levels that these are undetectable in our assay system.

Many genes of PMBLs are not constitutively expressed, but can be induced by mitogens. In vivo, induced lymphocyte proliferation normally occurs outside of the blood and thoracic duct. In vitro, specific mitogens are able to trigger the conversion of resting lymphocytes into actively growing and dividing blasts [26]. Mitogen stimulation mimics the series of events which occur in vivo following stimulation of PMBLs by specific antigens.

A very effective stimulator of T cell activation is the plant lectin phytohemagglutinin (PHA). The effect of PHA on the biosynthesis and secretion of APP was assessed after culturing PMBL's for various times with PHA and [^{35}S]methionine. Fig. 1b clearly demonstrates that PHA strongly induces the production and secretion of APP. The addition of the T cell specific mitogen to the culture medium resulted in a rapid stimulation of APP biosynthesis. As shown in Fig. 1b, anti-FdAPP antiserum could immunoprecipitate a 120 kDa protein from the culture medium. The band at 120 kDa has the same electrophoretic mobility as secreted APP in serum. APP secretion is observed within 3 h after PHA stimulation of PMBLs. Secretion continues over the whole stimulation period, i.e. over 4 days. In contrast, analysis of cell lysates showed that cellular APP was first detected 12 h after addition of PHA. The im-

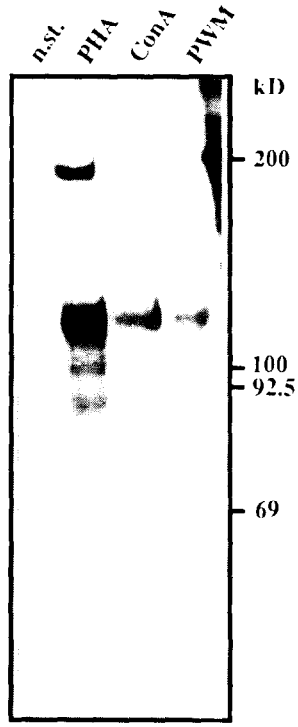


Fig. 2. Immunoprecipitation of APP from [^{35}S]methionine-labelled media of PMBLs stimulated with PHA, Con A, and PWM. Freshly isolated PMBLs were cultivated in the absence or in the presence of PHA, ConA or PWM. Biosynthetic labelling with [^{35}S]methionine was performed after 2 days of cultivation. APP was isolated by immunoprecipitation with anti-FdAPP antiserum. The immunoprecipitates were analysed as in Fig. 2.

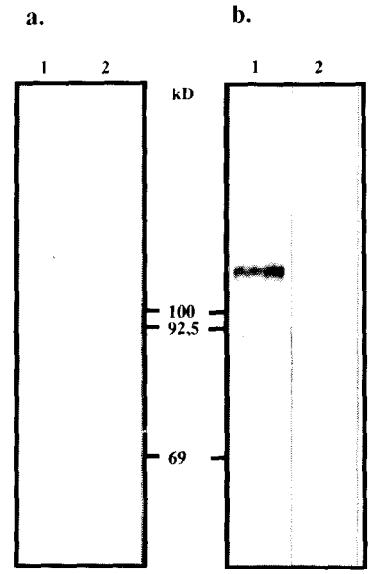


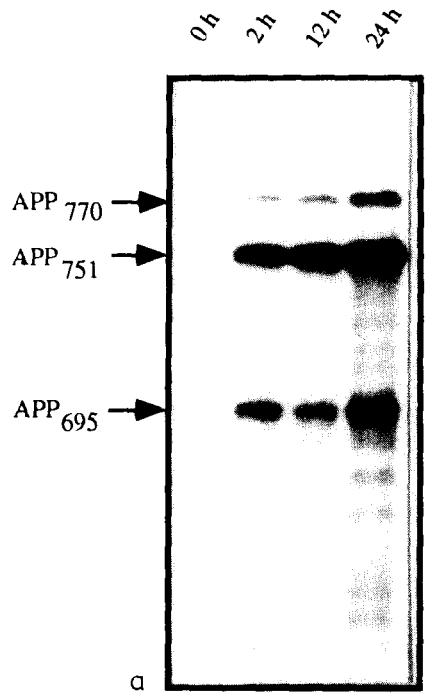
Fig. 3. Secreted APP in PHA-stimulated monocyte-depleted PMBLs. Monocytes were cultivated for 2 days in the presence of 1:5 diluted conditioned medium of PMBLs after stimulation with PHA for 3 days. Monocyte-depleted PMBLs were cultivated for 2 days with PHA. Biosynthetic labelling was initiated after the 2 day-period. APP of conditioned medium was analysed as in Fig. 2. The immunoprecipitate from conditioned medium of lymphocytes is shown in lane 1 and of monocytes in lane 2.

munoprecipitates showed bands in the range of 90–130 kDa which are molecular masses corresponding to intermediates or final products of APP biosynthesis of transmembrane APP forms described by Weidemann et al. [14]. Accumultion of intracellular APP is therefore a late event; during the first 12 h the majority of APP is secreted.

Initial experiments demonstrated that PMBLs could be triggered to secrete APP by stimulation with Con A, PWM and PHA. In addition, stimulation with PHA showed a 200 kDa band probably corresponding to an APP dimer since it is only seen if high concentrations of APP are present (Fig. 2). HPA seems to be the most potent inducer of APP biosynthesis. Because all three lectins stimulate T cell proliferation and lymphokine production and also trigger APP secretion of cultured PMBL's, it seemed likely that APP is a T cell product. As shown in Fig. 3, stimulation of freshly prepared, monocyte-depleted PMBLs also resulted in secretion of APP. Adherence-selected monocytes failed to produce APP after treatment with conditioned medium of PHA-stimulated PMBLs suggesting that APP secretion after PHA stimulation is indeed T cell specific. Preliminary results, however, suggest that monocytes also turn on APP biosynthesis during differentiation into macrophages.

To confirm the presence of APP mRNA in activated T lymphocytes and to examine the time course of expression of APP mRNA, human PMBLs were cultured with PHA for various periods of time. Total RNA from each group of cells was isolated and analyzed by a S1 nuclease protection assay [40]. Fig. 4a shows the induction of APP mRNA in PBMLs at various times after stimulation. Unstimulated cells had low to undetectable levels of APP mRNA, while PHA-activated cells expressed substantial levels of APP mRNA. The message appeared rapidly after stimulation. Elevations in the level of APP mRNA by a factor 16 are observed within 2 h after exposure to PHA. Maximum levels of gene expression (37-fold increase) are obtained within 24 h. After this time, the decrease of the APP message differs depending on cell preparation. In PHA-activated leucocytes the predominant APP gene splicing product is the APP_{751} mRNA (Fig. 4b). The dominance of APP_{751} mRNA is even more significant during stimulation. The results of the mRNA analysis are in accordance with the protein data. APP transcription, translation as well as secretion correlate with stimulation of the PMBLs.

An example for APP secretion by activated T lymphocytes in vivo is given in polymyositis, an inflammatory myopathy. This autoimmune disease is characterized by inflammatory and degenerative changes associated with endomysial infiltrates of cytotoxic lymphocytes. These infiltrates were strongly



	tot. APP_{751}	ratio $APP_{695/751/770}$
0	1	(-)+
2h	16	26/ 62/ 12
24h	37	18/ 69/ 13

b +not determined

Fig. 4. (a) Identification of APP transcripts in PMBLs by S1 nuclease protection assay. PMBLs were treated with PHA for 0 h (control, unstimulated PMBLs), 2 h, 12 h and 24 h. The corresponding fragments of APP_{695}, APP_{751} and APP_{770} mRNAs are indicated by arrows. (b) Relative abundance of total APP_{751} and ratio of APP_{695} to APP_{751} to APP_{770}.

APP-positive when immunostained with the monoclonal anti-APP antibody 22C11 (Fig. 5).

4. DISCUSSION

We demonstrate for the first time that stimulated human mononuclear leucocytes produce APP and might be a source of circulating serum APP. In contrast to different cell lines that constitutively synthesize and secrete APP, secretion of APP was undetectable in resting PMBLs. We have demonstrated that the APP biosynthesis of human mononuclear blood leucocytes is highly increased following induction with the T cell specific mitogens phytohemagglutinin, concanavalin A or pokeweed mitogen. Of these, PHA has the most potent stimulatory activity. It seems likely that APP is a T cell product since all tested mitogens stimulate T cell proliferation and lymphokine production [27,28]. PHA activation resulted in a maximum 37-fold increase of APP mRNA levels and secretion of APP within 24 h. Resting T cells were found to have nearly undetectable levels of APP mRNA. Finally, APP appears to be produced in vivo by cytotoxic lymphocytes which are a constant histological finding in muscle tissue of patients with polymyositis. Using immunohistochemistry with a monoclonal APP-antibody, we found strong immunoreactivity in a majority of these muscle-infiltrating lymphocytes.

The early APP gene response and the rapid secretion of APP at a time when cells are induced to proliferate may indicate a role of APP associated with T cell function. In general, T cells mediate their responses both by cell/cell contact and by secreted lymphokines. This process is regulated in part via lymphokine feedback circuits (for a review see [29]). The production of APP by T lymphocytes could therefore play a role in regulation of lymphocyte function or in the interaction of T cells with other cell types. The latter possibility is more likely since a general function of APP in cell/cell and cell/matrix interactions has been proposed for neuronal and non-neuronal APP [14,30–33].

Freshly isolated human peripheral blood leucocytes are arrested in the G_0-phase of the cell cycle. Upon stimulation of resting lymphocytes, T cells enter the G_1-phase of the cell cycle. The physiological mechanism can be mimicked by mitogenic plant lectins such as PHA. Lymphocytes respond to it by the production of de novo gene transcripts, e.g. proto-oncogenes, transferrin receptor and critical components of T cell growth as interleukin 2 (IL-2) and IL-2 receptor [34,35]. The rapid induction of APP biosynthesis in response to a stimulus is comparable with the time course of IL-2 induction [35]. This suggests that PHA stimulates lymphocyte synthesis of APP by a mechanism similar to the one which regulates the synthesis of early-response gene products such as IL-2. Corresponding protein-binding regions are present in the promoter sequence of the APP gene as described by Salbaum et al. [36]. Among others, the APP promoter includes heptamer sequences which resemble the consensus binding site of the transcription factor AP-1. The products of the oncogenes *v-jun* and *c-fos* have the ability to bind to this consensus sequence [37,38]. The actual control of transcription of the IL-2 gene appears to be regulated by a transcription enhancer in the 5′-flanking region of the interleukin-2 gene. There are defined regions within this 5′-flanking region to which particular nuclear factors may bind in order to activate transcription. Interestingly, the enhancer region of the IL-2 gene contains also an AP-1 binding site [39]. It has also been shown that the *c-fos* gene product is present in appropriately stimulated peripheral blood T cells [34].

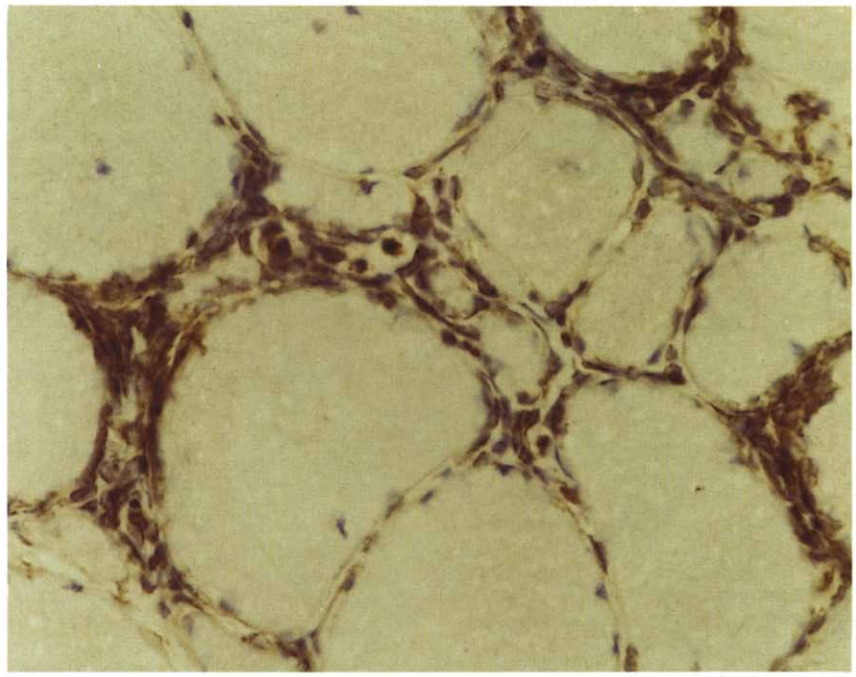


Fig. 5. APP expression in lymphocytes in vivo. Transverse section of a muscle biopsy obtained from a 58-year-old female patient with polymyositis. Atrophic and hypertrophic muscle fibers are seen, the endomysium is filled with lymphocytes that are strongly immunoreactive for APP. The periphery of some muscle fibers is invaded by cytotoxic APP-positive lymphocytes. Magnification ×200; counterstain with hematoxylin.

The presence of activated AP-1 has been suggested. Both the interleukin-2 gene and the APP gene are attractive candidates for *c-fos* or AP-1 directed stimulation.

Our results suggest that APP may play an important role in the construction of immunological networks and the differentiation of T cells. Thus, T cells may be an ideal model system to study the biological function of secretory APP.

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